DNA Damage-Induced RPA Focalization is Independent of γ-H2AX and RPA Hyper-Phosphorylation

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Abstract   Replication protein A (RPA) is the major eukaryotic single stranded DNA binding protein that plays a central role in DNA replication, repair and recombination. Like many DNA repair proteins RPA is heavily phosphorylated (specifically on its 32 kDa subunit) in response to DNA damage. Phosphorylation of many repair proteins has been shown to be important for their recruitment to DNA damage-induced intra-nuclear foci. Further, phosphorylation of H2AX (γ-H2AX) has been shown to be important for either the recruitment or stable retention of DNA repair proteins to these intra-nuclear foci. We address here the relationship between DNA damage-induced hyper-phosphorylation of RPA and its intra-nuclear focalization, and whether γ-H2AX is required for RPA's presence at these foci. Using GFP-conjugated RPA, we demonstrate the formation of extraction-resistant RPA foci induced by DNA damage or stalled replication forks. The strong DNA damage-induced RPA foci appear after phosphorylated histone H2AX and Chk1, but earlier than the appearance of hyper-phosphorylated RPA. We demonstrate that while the functions of phosphoinositol-3-kinase-related protein kinases are essential for DNA damage-induced H2AX phosphorylation and RPA hyper-phosphorylation, they are dispensable for the induction of extraction-resistant RPA and RPA foci. Furthermore, in mouse cells genetically devoid of H2AX, DNA damage-induced extraction-resistant RPA appears with the same kinetics as in normal mouse cells. These results demonstrate that neither RPA hyper-phosphorylation nor H2AX are required for the formation in RPA intra-nuclear foci in response to DNA damage/replicational stress and are consistent with a role for RPA as a DNA damage sensor involved in the initial recognition of damaged DNA or blocked replication forks. J. Cell. Biochem. 99: 1452–1462, 2006.   © 2006 Wiley-Liss, Inc.

Key words: replication protein A (RPA); DNA damage; replicational stress; PI3 kinase; γ-H2AX; GFP

Replication protein A (RPA) is the major eukaryotic single-stranded DNA (ssDNA) binding protein that also has high affinity for damaged double-stranded DNA (dsDNA) [Patrick and Turchi, 1999; Lao et al., 2000]. There are three subunits in each RPA molecule, 70, 32, and 14 kDa (RPA70, RPA32, and RPA14). RPA is an essential DNA replication factor that plays critical roles in DNA recombination and most types of DNA repair synthesis [Wold, 1997; Iftode et al., 1999]. Genetic studies from budding yeast suggest that RPA is involved in DNA replication checkpoint functions [Longhese et al., 1996]. In human cells, down regulation of either RPA70 or RPA32 with small interfering RNAs (siRNA) results in prolonged S phase, spontaneous DNA strand breaks, G2/M arrest and cell death [Dodson et al., 2004]. These observations suggest that RPA is required for the maintenance of genome stability. Exactly what roles RPA plays in these functions is still unclear. In vitro studies suggested that RPA is required for the recruitment of several DNA damage checkpoint sensors, including ATR kinase, Rad17-RFC2-5 and Rad9-Rad1-Hus1 complexes, to the sites of damaged DNA or stalled replication forks [Ellison and Stillman, 2003; Zou and Elledge, 2003; Zou et al., 2003; Unsal-Kacmaz and

Sancar, 2004]. Hence, the multi-functional RPA also appears to act as a DNA damage sensor.

During G1, S, and G2 phases of the cell cycle, RPA is nuclear, with the majority of the RPA in the nucleosol, and readily diffusable upon disruption of the nuclear membrane [Dimitrova and Gilbert, 2000; Loo and Melendy, 2000]. During S phase of the cell cycle, a small portion of the RPA becomes associated with the nuclear matrix to form intranuclear foci, which correspond to sites of DNA replication [Brenot-Bosc et al., 1995; Krude, 1995; Dimitrova and Gilbert, 2000]. RPA foci can also be found in cells with damaged DNA. DNA damage-induced RPA foci co-localize with foci of several recombination and repair proteins, including: Rad 51, Rad 52, XPA, PCNA, Brca1, γ-H2AX, and Werner's helicase [Gasior et al., 1998; Golub et al., 1998; Raderschall et al., 1999; Sakamoto et al., 2001; Choudhary and Li, 2002; Liu et al., 2003b]. These foci likely reflect RPA's function in various DNA repair pathways. On sites of DNA replication in live cells, the turn over of RPA is relatively fast [Sporbert et al., 2002]. This is possibly due to DNA synthesis that continuously recruits nucleosolic RPA to newly exposed helicase-unwound ssDNA, while RPA is simultaneously released from the same sites during Okazaki fragment elongation. The turn over rate of RPA at sites of DNA damage is unknown.

RPA is a phospho-protein. In *S. cerevisiae*, RPA70 is phosphorylated in response to DNA damage by Mec1 kinase, a functional ortholog of mammalian ATR kinase [Brush and Kelly, 2000; Kim and Brill, 2003; Bartrand et al., 2004]. It is unclear whether this modification has any effect on RPA's functions [Kim and Brill, 2003]. The 70 kDa subunit of human RPA has recently been shown to be phosphorylated in crude cell extracts stimulated with DNA fragments, and this appears to affect RPA's function [Nuss et al., 2005; Patrick et al., 2005]. The 70 kDa subunit of RPA purified from human cells can be phosphorylated by both ATR and Chk1 kinases in vitro [Liu et al., 2006]. As was found for yeast RPA, phosphorylation of human RPA70 by ATR does not appear to have any observable effect on RPA's function; however, RPA70 phosphorylated by Chk1 shows reduced ssDNA binding activity [Liu et al., 2006]. RPA32 is cell cycle phosphorylated and hyper-phosphorylated in response to DNA damage or apoptosis [Treuner et al.,

1999; Binz et al., 2004]. Several kinases are involved in the phosphorylation of RPA32. Cyclin-dependent kinases and Ime2 kinase are responsible for cell cycle- or meiosis-regulated phosphorylation of RPA32; and three phosphoinositol-3-kinase-related protein kinases (PI3Ks), ataxia telangiectasia mutated (ATM) kinase, ATM-Rad3-related protein kinase (ATR) and DNA-dependent protein kinase (DNA-PK) contribute to DNA damage-induced RPA32 hyper-phosphorylation [Pan et al., 1994; Brush et al., 2001; Oakley et al., 2003; Binz et al., 2004; Clifford et al., 2004]. It is not known which kinase is responsible for the hyper-phosphorylation of RPA32 during apoptosis. Phosphorylation of RPA on its 32 kDa subunit by purified kinases did not change RPA's functions in in vitro assays [Pan et al., 1995; Henricksen et al., 1996]. A GFP-RPA mutant was created where the PI3K-targeted serines and threonines on RPA32 were replaced by charged aspartic acid residues to mimic the hyper-phosphorylated form of RPA. This GFP-RPA mutant is able to associate with intra-nuclear DNA damage foci, but not to DNA replication foci, suggesting one possible mechanism for regulating RPA's function in response to DNA damage [Vassin et al., 2004].

As has been reported for other DNA damaging agents, we have shown that RPA focalizes and is hyper-phosphorylated in response to treatment of cells with two highly specific DNA damaging agents, adozelesin and C-1027 [Liu et al., 2000, 2001, 2003a,b]. Adozelesin binds to the minor groove of A/T-rich DNA sequences and alkylates the N3 of adenine at the 3′-end of the binding site [Yoon and Lee, 1998]. Adozelesin-induced RPA and γ-H2AX (histone H2AX phosphorylated at serine 139; [Redon et al., 2002]) focus formation and RPA32 hyper-phosphorylation are only found in S phase cells with active DNA replication fork progression [Liu et al., 2003a]. This response is similar to that reported for UV DNA damage, but is likely more homogenous due to the more specific nature of the DNA lesions produced by adozelesin. In contrast to adozelesin, we have shown that the DNA strand scission agent, C-1027, is capable of inducing γ-H2AX focus formation and RPA32 hyper-phosphorylation in virtually all treated cells [Liu et al., 2003a]. C-1027's ability to preferentially produce dsDNA breaks makes it an important mimetic for ionizing radiation [Dziegielewski and Beerman, 2002]. Due to the

different requirement for DNA replication, the patterns of adozelesin and C-1027 induced RPA foci are quite different [Liu et al., 2003a]. This pair of drugs is used in this study to evaluate the DNA replication-dependent and -independent induction of RPA focalization and hyper-phosphorylation in treated cells.

In this report we evaluate the DNA damage-induced RPA focalization and RPA32 hyper-phosphorylation and their relationship to PI3K activities. To understand the role of RPA in early cellular responses to DNA damage, human 293 cells expressing GFP-RPA were used to monitor DNA damage-induced RPA focalization in live cells. The appearance of focalized γ-H2AX is one of the earliest DNA damage responses reported [Redon et al., 2002]. Furthermore, the presence of H2AX is essential for focalization of many early DNA damage response proteins, like Mre11, NBS1, and 53BP1 [Bassing et al., 2002; Celeste et al., 2002; Ward et al., 2003]. Hence, we compared the appearance and inter-relatedness of γ-H2AX with RPA hyper-phosphorylation and focalization. Our results suggest that RPA is involved in multiple steps in the early cellular responses to DNA damage and DNA replication fork blockage.

MATERIALS AND METHODS

Chemicals and Antibodies

4,6-diamidino-2-phenylindole (DAPI) and wortmannin were purchased from Sigma. Adozelesin in dimethylacetamide (2 mg/ml) and C-1027 diluted in water (2 mg/ml) were kindly provided by Dr. Terry Beerman. Monoclonal antibodies against γ-H2AX and human Chk1 were purchased from Upstate Biotech and Santa Cruz Biotech, respectively. The monoclonal and affinity purified polyclonal antibodies against RPA32 were as described [Din et al., 1990; Liu et al., 2001]. Fluorescein conjugated goat anti-mouse and Alexa 568-conjugated goat-anti rabbit antibodies were purchased from Vector Laboratory Inc. and Molecular Probes, Inc., respectively. Tissue culture dishes with cover glass bottoms (FluoroDish) were from World Precision Instruments, Inc.

Cell Culture

Monolayer cultured human 293 and HeLa cells and mouse cells with or without genetically knock-out H2AX (H2AX$^{(+/+)}$ and H2AX$^{(-/-)}$; gifts from Dr. Nussenzweig) were maintained in DMEM with 10% FBS. Plasmid pEGFP-RPA70 was cloned by inserting the full length RPA70 open reading frame at the 3' end of the enhanced green fluorescent protein coding sequence in the pEGFP-C1 expression plasmid (CLONTECH). This expression plasmid was delivered into 293 cells using calcium phosphate co-crystalization transfection protocal [Jordan et al., 1996]. pEGFP-RPA70 transfected 293 cells were maintained in HEPES-buffered DMEM (Invitrogen) with 10% FBS. Fluorescence activated cell sorting (FACS) was performed by Dr. R. Kelleher at UB Imaging Core Facility with FACSTAR Cell Sorter (BD Biosciences).

Indirect Immunofluorescent Staining and Immunoblotting

Monolayer cultured cells (1×10^6) treated with DNA damaging drugs as indicated were rinsed with PBS and harvested by trypsinization. Cells were permeabilized in PBS containing 0.25% Triton X-100 for 5 min at room temperature and separated into two halves. One half of the cells was pelleted ($1,000g$, 3 min), and soluble fractions (S) were transferred to fresh tubes and the pellets (extraction-resistant fractions; ER) were resuspended in the same volume of PBS. Total protein from an equal number of cells ($\sim 2 \times 10^4$) was mixed with SDS sample buffer (20 mM Tris-HCl pH 7.5; 2% SDS; 1 M 2-mercaptoethanol) and resolved by electrophoresis on 12.5% (w/v) SDS-polyacrylamide gels and transferred to Hybond-P membrane using NovaBlot (Amersham Pharmacia Biotech) as per the manufacturer's instructions. The membranes were probed with monoclonal antibodies against GFP, RPA70, RPA32, Chk1, or γ-H2AX at room temperature for 1 h. Peroxidase-conjugated goat anti-mouse IgG (Pierce) was used as the secondary antibody. The membranes were then incubated with Supersignal ECL reagent (Pierce) and exposed to X-ray film.

The other half of cells was spun onto a poly-L-Lysine-coated rounded cover glass through 1 M sucrose in PBS and fixing with 3% paraformaldehyde in PBS. The fixed cell nuclei were pre-blocked with 10% normal goat serum and 3% BSA in PBS, and incubated with anti-γ-H2AX monoclonal antibody and antigen-purified polyclonal antibody against RPA32 (in PBS

containing 3% normal goat serum, 3% BSA and 0.5% Triton X-100) at room temperature for 1 h. Fluorescein-conjugated goat anti-mouse and Alexa 568-conjugated goat anti-rabbit antibodies were then used as secondary antibodies at room temperature for 1 h. DAPI (2 mM) was used to counterstain DNA. RPA and γ-H2AX foci were examined using a Leiz Orthoplan 2 epifluorescent microscope with a SPOT-RT digital camera, or a Bio-Rad MRC-1024 confocal imaging system. Adobe Photoshop was used for image processing and printing.

RESULTS AND DISCUSSION

DNA Damage-Induced RPA Focus Formation in Live Cells

We created a GFP-RPA70 expression vector to generate GFP-RPA in human 293 cells. Although GFP-RPA32 had been previously constructed [Sporbert et al., 2002; Vassin et al., 2004], we favored the use of a GFP-RPA70 version, as the GFP signal from GFP-RPA70 would be present exclusively in the form of the RPA heterotrimer. This is supported by two findings. It has been shown that RPA70 in the absence of RPA32 is quickly degraded in vivo [Dodson et al., 2004]. Conversely, RPA32 free of RPA70 can be detected in RPA70 siRNA-treated cells as well as in 293 cells [Loo and Melendy, 2000; Dodson et al., 2004]. Over expression of GFP-RPA70 would therefore efficiently produce GFP-RPA heterotrimer without concern for the presence of any free GFP-RPA70 monomer.

Human 293 cells transfected with the GFP-RPA70 expression plasmid were selected with G418 for 10 passages, harvested and sorted by FACS to collect cells expressing intermediate levels of GFP. As shown in Figure 1, GFP-

Fig. 1. GFP-RPA70 complexes with RPA32 in transfected cells. Whole cell extracts from 293 cells transfected with pEGFP-RPA70 (**lane 1**) were applied to a 5-ml 5–20% sucrose density gradient for separation by sedimentation. Selected fractions containing heterotrimeric RPA as well as possible individual subunits were analyzed by immunoblotting. Fraction #5 is near the bottom and #12 near the top of the gradient. Monoclonal antibodies against RPA70 and RPA32 were used to detect RPA. Each RPA subunit is indicated to the left of the panel.

RPA70 represented about 5% of total RPA70 in the transfected cells (lane 1). When separated on a 5–20% sucrose density gradient, virtually all of the GFP-RPA70 and the endogenous RPA70 co-migrated with RPA32 (lanes 3–5) and most likely formed heterotrimeric RPA complexes, as shown previously [Loo and Melendy, 2000]. As noted previously for untransfected cells, a small fraction of the RPA32 sediments more slowly than the RPA trimer, free of RPA70 (Fig. 1, fractions 11 and 12, lanes 8–9) [Loo and Melendy, 2000]. To demonstrate that this GFP-RPA remains responsive to DNA damage focalization in vivo, GFP-RPA-expressing 293 cells were treated with the DNA damaging agent adozelesin for 90 min. Cells were harvested and lysed with PBS containing 0.25% Triton X-100 and divided into two halves (Materials and Methods). One half of cells was separated into the soluble (cyto/nucleosolic = S) and extraction-resistant (chromatin/nuclear matrix = ER) fractions by centrifugation, and analyzed via immunoblotting using antibodies specific to GFP, RPA70 and RPA32. The nuclei from the second group were spun through a sucrose cushion onto a cover glass, and the permeabilized nuclei were examined using fluorescence microscopy. In adozelesin-treated cells, the same proportion of RPA and GFP-RPA became extraction resistant (ER; Fig. 2A, lane 4), and GFP-RPA formed intra-nuclear foci (Fig. 2B). Consistent with our results with untransfected cells, about 40% of the treated cells (those in S phase) showed GFP-RPA foci with patterns similar to the RPA foci induced by adozelesin treatment and visualized by indirect-immunostaining (data not shown) [Liu et al., 2003a]. These results indicate that this GFP-RPA is functional for intra-nuclear focalization in transfected 293 cells.

DNA damage-induced GFP-RPA focalization was then monitored in live cells. Transfected 293 cells cultured on cover glasses were treated with either the S-phase specific DNA damaging agent adozelesin, or the IR mimetic dsDNA break agent C-1027, and monitored continuously using an inverted confocal microscope inside a 37°C chamber. Continuous observation of the live cells following treatment showed that the earliest RPA foci were seen between 20 and 30 min after addition of C-1027 (Fig. 3). After 90 min of treatment, only about 1% of adozelesin-treated cells and less than 20% of
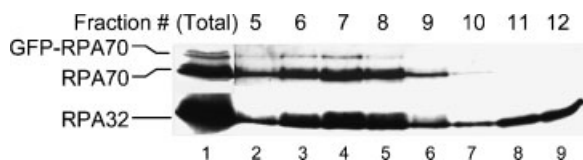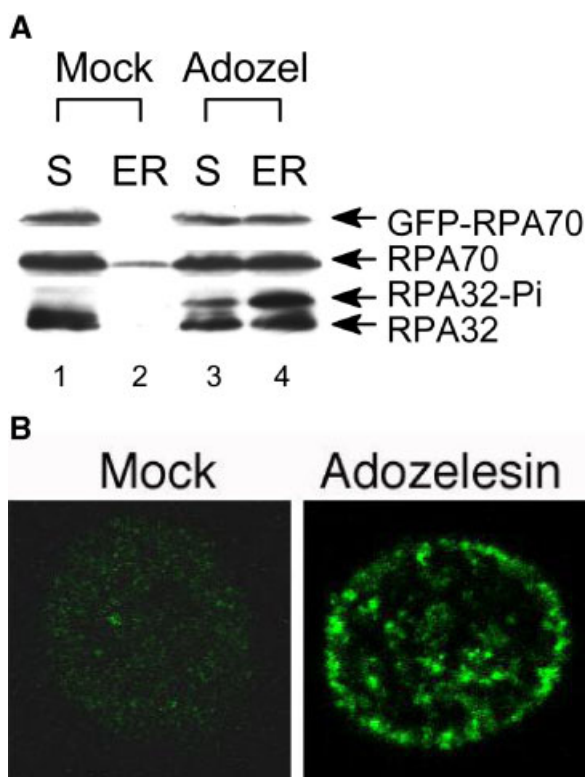
Fig. 2. GFP-RPA is functional in damage-induced focalization in vivo. Monolayer cultured 293 cells expressing GFP-RPA were either mock-treated or treated with 20 nM adozelesin for 90 min, harvested, permeabilized with PBS and 0.25% Triton X-100 and separated into two halves (see Materials and Methods). **A:** One half of the cells were used to prepare soluble (S) and extraction resistant (ER) fractions as described in the Materials and Methods, and analyzed by immunoblotting with specific antibodies to GFP, RPA70, and RPA32. **B:** The other half was layered on top of PBS with 1 M sucrose in six-well plates. Cell nuclei were spun onto cover glasses at the bottom of each well, fixed, subjected to immunostaining, and examined using confocal microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

C-1027-treated cells displayed a few bright green fluorescent spots (Fig. 3, note increase in foci of cell indicated by arrow and additional data not shown). Since more than 40% of adozelesin-treated cells and 90% of C-1027-treated cells show large numbers of extraction resistant RPA foci (see Figs. 4 and 6) [Liu et al., 2003a], this suggests that in the absence of permeabilization and washing away the nucleosolic RPA, only the very brightest foci are visible in these 2 μ confocal images. Rapid diffusion of the nucleosolic RPA may also contribute to this as fluorescence recovery after photo bleaching showed that the GFP signal at photo-bleached foci recovers fully at the first measured time point of 5 s (data not shown). The small number of bright spots detected may represent rare clusters of DNA damage-induced RPA foci or a region of extensively unwound DNA [Byun et al., 2005]. Clearly, there is a ready supply of nucleosolic RPA within cell nuclei which can rapidly associate with sites of DNA damage presumably to facilitate DNA repair. These results also demonstrate that DNA damage-induced intra-nuclear focalization occurs in live cells and is not a result of post harvest manipulation or fixation conditions.

DNA Damage-Induced RPA and γ-H2AX Focalization

The timing for DNA damage-induced RPA focus formation was further evaluated using immunostaining. Human 293 cells were treated with adozelesin or C-1027 for different lengths of time, lysed with Triton X-100 and separated into two halves as described above. Monoclonal antibody against RPA32 was used to detect soluble (S) and extraction resistant (ER) RPA in immunoblots (Fig. 4A; adozelesin-treated cells), and antigen-purified polyclonal antibody against RPA32 was used to stain RPA foci (Fig. 4B). Since γ-H2AX is known to be one of the earliest proteins to appear at sites of DNA damage or blocked replication forks, it was used as a timing marker. Extraction-resistant RPA and RPA foci appeared within 20 min in cells treated with either drug and reached maximal levels between 40 and 60 min (Fig. 4A, lanes 3–8, data not shown and Figure 4B, red), similar to that was found in live cells with GFP-RPA (Fig. 3). In these same cells, induction of γ-H2AX can be seen within 10 min of treatment (Fig. 4B, green) and occurs only in the chromatin/nuclear-matrix fraction (Fig. 4A, even number lanes). Considering the time required for the drug to diffuse into the cells and damage DNA, this is consistent with induction of γ-H2AX being one of the earliest responses to C-1027 and adozelesin treatment. This event is followed quickly by further recruitment of more RPA to the same sites.

PI3Ks are responsible for DNA damage-induced phosphorylation of serine 139 of H2AX, forming γ-H2AX. It is therefore reasonable to surmise that one or more of the PI3Ks are immediately activated and recruited to sites of damage for phosphorylation of H2AX. All three PI3Ks are able to phosphorylate RPA32 in vitro at multiple sites. We compared the timing of adozelesin- and C-1027-induced
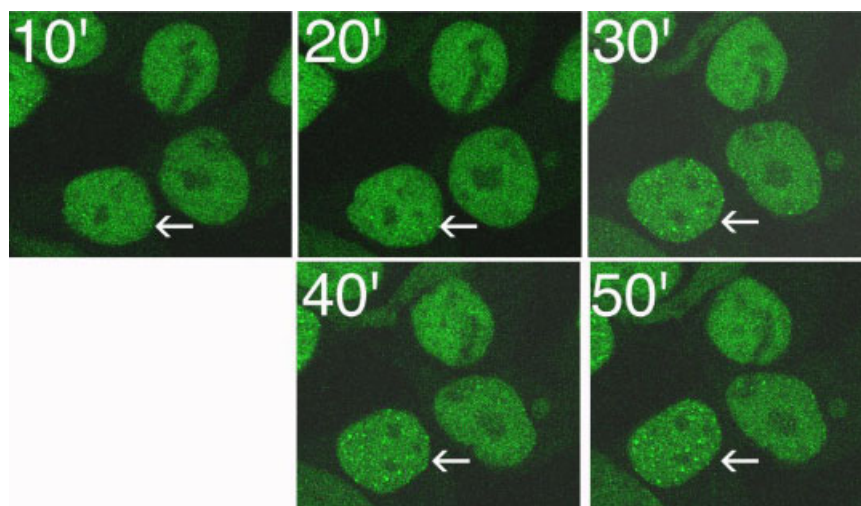
Fig. 3. GFP-RPA form foci in live cells treated with C-1027. Human 293 cells transfected with pEGFP-RPA70 were seeded on culture dishes with cover glass bottoms and treated with 1 nM C-1027. Cells were maintained at 37°C in a temperature-controlled chamber on an inverted confocal microscope. Images were taken at 10, 20, 30, 40, and 50 min after addition of C-1027 and processed using Adobe Photoshop. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hyper-phosphorylation of RPA32 to phosphorylation of H2AX and Chk1. As shown in Figure 5, the induction of γ-H2AX and phosphorylated Chk1 was detectable within 5 min of C-1027 or 20 min of adozelesin treatment. However, the appearance of hyper-phosphorylated RPA32 was substantially slower, about 40 and 90 min after the addition of C-1027 and adozelesin, respectively. It is not clear whether low levels of RPA32 phosphorylation, insufficient to cause a mobility shift or insufficient for detection, occurs at earlier time points. However, the delay in RPA focalization relative to the phosphorylation of H2AX and Chk1 (compare Figs. 3 and 4) suggests additional events or kinases are required prior to RPA32 hyper-phosphorylation.

Based on the results of these time course studies, we propose a possible mechanism for adozelesin-induced early checkpoint responses. RPA is an essential DNA replication factor present at each replication fork at a low level [Sogo et al., 2002]. Collisions of either helicases or DNA polymerases with adozelesin adducts will result in stalled DNA replication forks [Maine et al., 1992]. Based on in vitro studies, we anticipate that ATR is immediately recruited to the sites of blocked replication forks through interaction with RPA [Zou and Elledge, 2003; Ball et al., 2005] and would then phosphorylate Chk1 and H2AX [Shechter et al., 2004]. Other checkpoint factors, such as the

Rad17 and Rad9-Rad1-Hus1 complexes, might be recruited at the same time [Ellison and Stillman, 2003; Zou et al., 2003]. The delayed appearance of stronger RPA foci at the same sites [Liu et al., 2003a,b] suggests a secondary event that possibly creates more ssDNA [Byun et al., 2005] to be bound by more RPA. We and others have shown that phosphorylation of RPA32 by ATR in vitro is stimulated preferentially by longer stretches of ssDNA [Zou and Elledge, 2003; Bartrand et al., 2004; Kumagai et al., 2004; Liu et al., 2006]. This likely explains the further delay before the appearance of large amounts of hyper-phosphorylated RPA32.

Treatment of cells with the DNA scission agent, C-1027, resulted in earlier and stronger H2AX phosphorylation, Chk1 phosphorylation, and hyper-phosphorylation of RPA32 than with adozelesin (Fig. 5). However, it is interesting to note that the appearance of strong RPA foci induced by these two drugs occurs at about the same time (Fig. 4B). This may be due to C-1027 treatment preferentially inducing double strand breaks on chromosomal DNA that is not already occupied by RPA, as opposed to adozelesin treatment which only induces RPA foci at sites of DNA replication, where RPA is already present [Liu et al., 2003a]. It is possible that a small amount of RPA can be quickly associated with C-1027 damaged sites and then brings in PI3Ks for downstream signaling. Alternatively, PI3Ks may be recruited by other
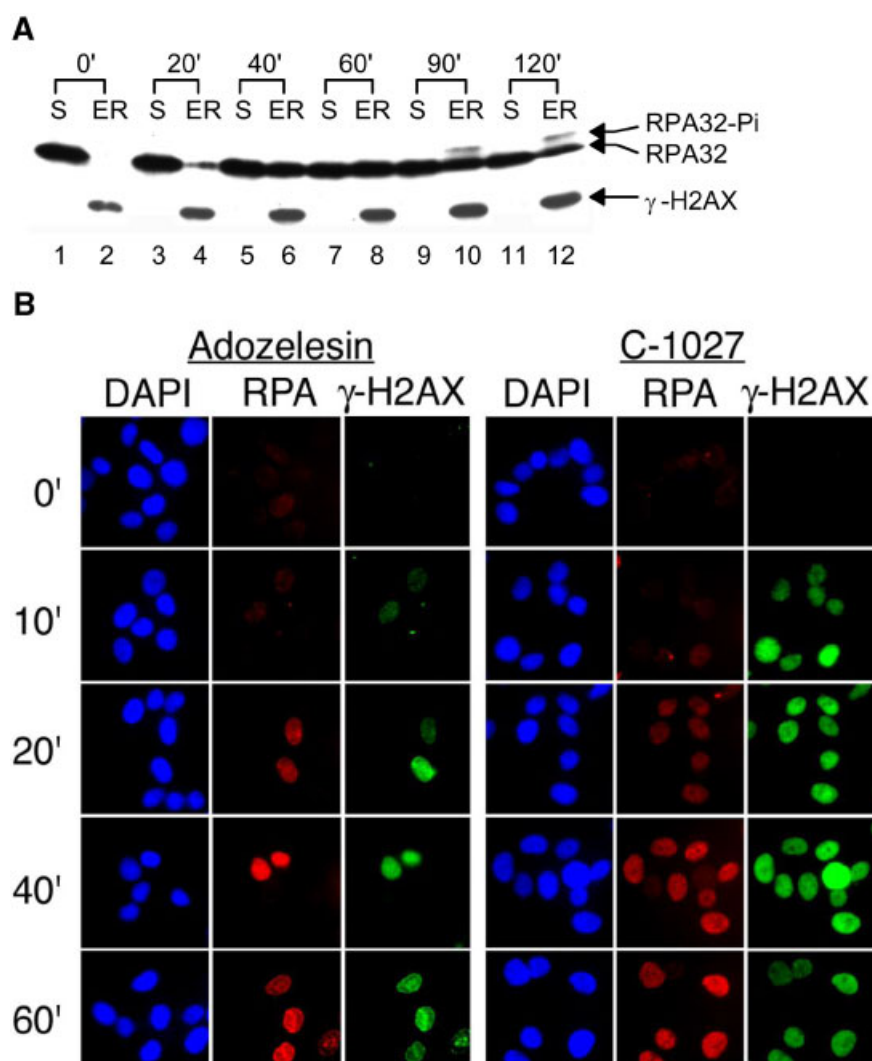
Fig. 4. RPA focus formation is an early response to DNA damage. HeLa cells treated with 20 nM adozelesin or 1 nM C-1027 for the length of time indicated on (**A**) top or (**B**) the right side of the panel, permeabilized and separated into two halves as in Figure 2. A: One half of the adozelesin-treated cells were used to prepare soluble (S) and extraction resistant (ER) fractions, and analyzed by immunoblotting with specific antibodies to RPA32 and γ-H2AX. Each protein is labeled on the right side of the panel.

B: The other half of adozelesin-treated cells (**left panels**) or C-1027-treated cells (**right panels**) was spun onto cover glasses, fixed, and subjected to stain for total DNA with DAPI (blue) and immunostaining for RPA (red) and γ-H2AX (green). The images were captured by a fluorescent microscope with a 100X objective lens under oil immersion, and processed using Adobe Photoshop. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

intermediates (for example, Mre11/Rad50/Nbs1 complex, Ku70/80, and Mdc1) that are independent of RPA [Falck et al., 2005; Peng and Chen, 2005; Stiff et al., 2005]. However, as noted in the model described above, the strong RPA foci that appear after the induction of γ-H2AX are most likely due to a secondary event that activates a nuclease and generates more extensive ssDNA at sites of damage. We therefore investigated whether the appearance of the adozelesin and C-1027-induced strong RPA foci are dependent on PI3K function or H2AX.

RPA Focus Formation is Independent of PI3-Family Kinase Activity

We have previously shown that DNA damage-induced RPA focus formation is not affected when the function of either DNA-PK or ATR is blocked [Liu et al., 2001, 2003a]. However, it is known that the PI3-family kinases have a high level of functional redundancy and can often compensate for each other's absence. Wortmannin, which blocks phosphorylation by all three PI3Ks, was therefore used to treat 293 cells
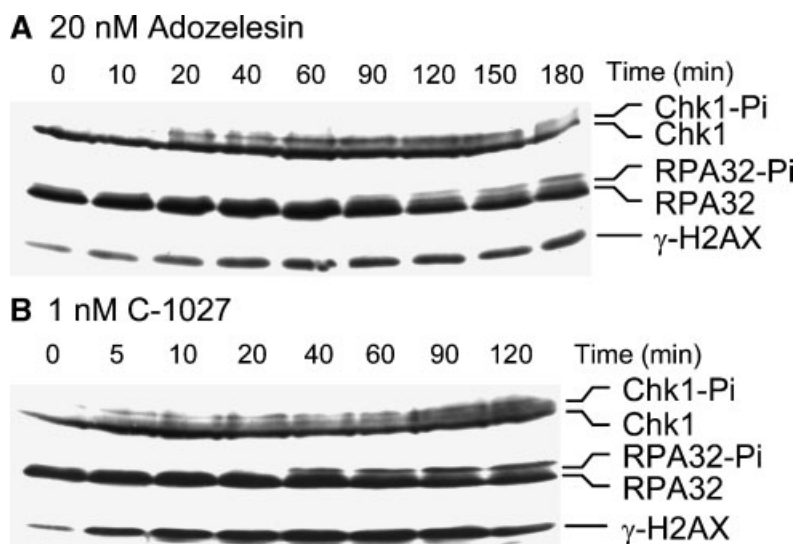
Fig. 5. Adozelesin and C-1027 induced phosphorylation of H2AX and Chk1 occurs earlier than RPA focus formation and RPA32 hyper-phosphorylation. HeLa cells treated with (**A**) 20 nM adozelesin for 0–180 min, or (**B**) 1 nM C-1027 for 0–120 min were harvested, resuspended in denaturing SDS sample buffer and analyzed by immunoblots with monoclonal antibodies to Chk1, RPA32, and γ-H2AX. Migration of proteins is indicated at the right side of each panel.

prior to the addition of either adozelesin or C-1027. The treated cells were separated into two halves as described and analyzed for RPA extraction resistance, hyper-phosphorylation and focalization. Wortmannin alone did not change RPA's extractability (Fig. 6A, lanes 1–4) or induce RPA or γ-H2AX foci (Fig. 6B, second row). Wortmannin did block RPA32 hyper-phosphorylation and dramatically reduced γ-H2AX induction and focalization following treatment of cells by C-1027 or adozelesin (Fig. 6A, compare lanes 5 and 6 to 7 and 8; Fig. 6B and C green, second columns). However, DNA damage-induced extraction-resistant RPA or RPA foci were not affected by wortmannin (Fig. 6A, compare lanes 6 and 8; Fig. 6B and C, red, first columns). These results indicate that PI3K activity is required for the induction of γ-H2AX and hyper-phosphorylation of RPA32, consistent with previous studies [Paull et al., 2000]. Conversely, DNA damage-induced intra-nuclear focalization of RPA is independent of PI3K activities. These data indicate that RPA focalization is independent of both RPA hyper-phosphorylation and γ-H2AX. Further, they suggest that if creation of longer ssDNA at sites of stalled replication forks or damaged DNA for further DNA repair processes [Byun et al., 2005] is required for RPA recruitment, that this process does not require PI3K activity or H2AX phosphorylation.

DNA Damage-Induced Extraction Resistant RPA Does not Require H2AX

H2AX is an integral part of normal chromatin structure. Stable intra-nuclear focalization of several DNA damage proteins were found to require the presence of H2AX [Fernandez-Capetillo et al., 2004]. Since RPA foci also appear after γ-H2AX, and since we had shown that RPA focalization did not require phosphorylation of H2AX, we evaluated RPA's DNA damage-induced extraction resistance in mouse cells where H2AX was genetically deleted. (Immunostaining of RPA in these mouse cells could not be carried out as no currently available antibodies immunostain murine RPA.) As shown in Figure 7, from 30 to 90 min of C-1027 treatment, the levels of extraction-resistant RPA and hyper-phosphorylated RPA32 were virtually indistinguishable in H2AX-deficient versus wild-type mouse cells, indicating that H2AX is not required for RPA focalization in response to DNA damage.

SUMMARY

RPA is known to bind to many types of DNA including ssDNA, distorted dsDNA, dsDNA with lesions on one strand, and DNA ends [Patrick and Turchi, 1999; Lao et al., 2000; Binz et al., 2004]. The abundance of RPA in nuclei, the presence of low levels of RPA at replication
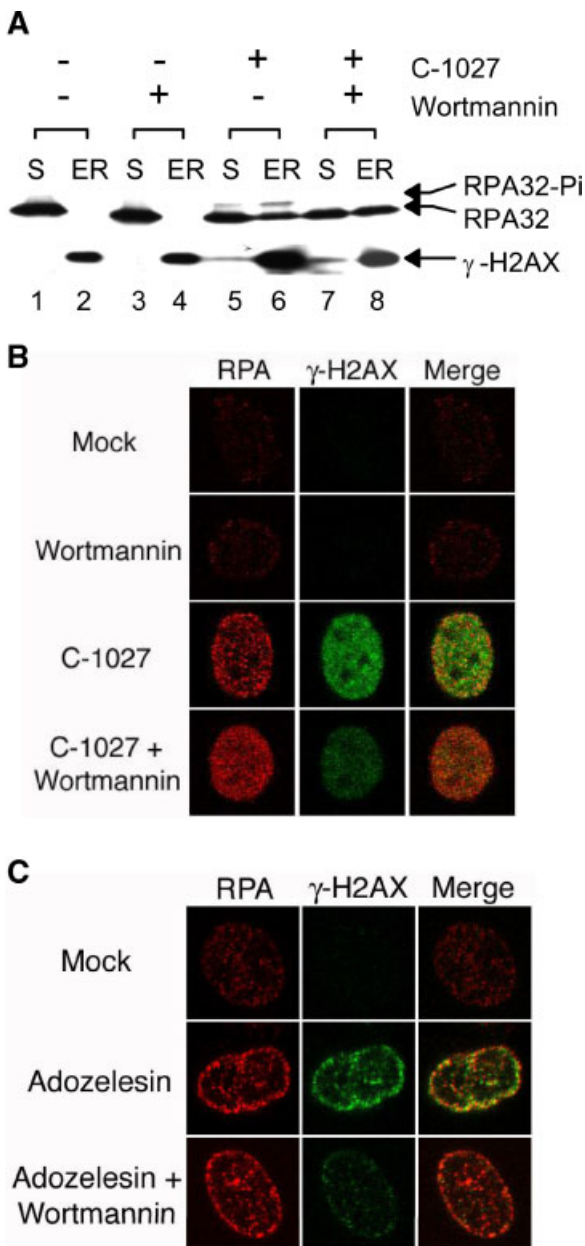
Fig. 7. γ-H2AX is not required for induction of extraction-resistant RPA or RPA hyper-phosphorylation. Cells from H2AX-deficient mice (**lower panel**) or control mice (**top panel**) were treated with 1 nM C-1027 for 0, 30, 60, or 90 min before harvesting. The cells were then permeabilized and separated into soluble (S; odd number lanes) and extraction resistant (ER; even number lanes) fractions as above, and analyzed by immunoblotting with monoclonal antibody against human RPA32. Migration of RPA32 and hyper-phosphorylated RPA32 is indicated at the right side of each panel.

forks, and the ability of RPA to recruit the DNA damage sensor ATR and Rad 17 to ssDNA, combine to suggest that RPA plays a surveillance function in the very earliest stages of DNA damage responses, DNA damage recognition. As a key player in the majority of DNA repair and recombination pathways, more nucleosolic RPA and other DNA repair factors are recruited to sites of DNA damage or stalled replication forks. As DNA is repaired, this facilitates the recovery of cells from the DNA damage response pathways and allows resumption of DNA synthesis. When DNA damage is not readily repairable, to prevent propagation of mutations and preserve the genome integrity of the organism, cells with persistent DNA damage are induced to undergo apoptosis. RPA's association with each step of the process further suggests it functions as a major guardian of genome stability.

Fig. 6. Wortmannin blocks DNA damage-induced phosphorylation of RPA32 and H2AX but not RPA focus formation. HeLa cells were treated with 10 μM wortmannin for 30 min before the addition of 1 nM C-1027 or 20 nM adozelesin. After 45 min of treatment cells were permeabilized and separated into two halves as in Figure 2. **A**: One half of C-1027-treated cells were used to prepare soluble (S) and extraction resistant (ER) fractions, and analyzed by immunoblots with specific antibodies to RPA32 and γ-H2AX. Migration of each protein is labeled on the right side of the panel. The other half of (**B**) C-1027-treated or (**C**) adozelesin-treated cells was spun onto cover glasses, fixed, and subjected to immunostaining for RPA (red) and γ-H2AX (green). The images were captured by a confocal microscope using a 60X objective lens under oil immersion, and processed using Adobe Photoshop. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
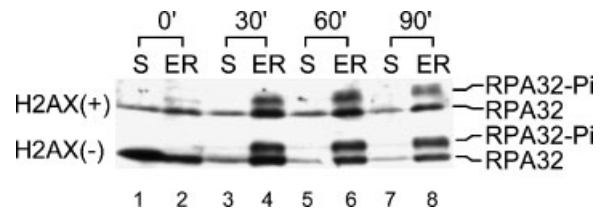
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